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Stefanelli et al. Biochemical journal (England) May 1, 2000, 347 Pt. 3, p875-80.

Sakagami et al. Anticancer Research (Greece), Jan-Feb. 2000, 20 (1A), p265-70.

Ray et al., American journal of physiology, Cell physiology (US), Mar. 2000, 278 (3), pC480-9.

Bock et al. Radiation research (US), Dec. 1999, 152 (6), p604-10.

Dai et al. Cancer research (US), Oct. 1, 1999, 59 (19), p4944-54.

Bratton et al. Jo. of biological chemistry (US), Oct. 1, 1999, 274 (40), p28113-20.

Palyi et al. Anti-cancer drugs (England), Jan 1999, 10 (10, p103-11.

Li et al. Am. journal of physiology , April 1999, 276 (4 Pt. 1), pC946-54.

Ray et al. Am. journal of physiology, Mar. 1999, 276 (3 Pt. 1) pC684-91.

Das et al. Oncology Research (US), 1997, 9 (11-12), p565-72.

Monti et al.. Life Sciences (England), 1998, 62 (9), p799-806.

Lin et al., Experimental cell research, (US), Nov. 25, 1997.

Tome et al. biochemical Journal (England) Dec. 15, 1997, 328 (Pt. 3), p847-54.

Hu et al., Biochemical journal (England), Nov. 15, 1997, 328 (Pt. 1), p307-16.

Tome et al. biological signals (Switzerland), May -Jun 1997, 6 (3), p150-6.

Taguchi et al., Cell biochemistry and function (England), Mar 2001, 19 (1), p19-26.

Camon et al. neurotoxicology (US), Fall 1994, 15 (3), p759-63.

Shinki et al., Gastroenterology (US), Jan 1991, 100 (1), p113-22.

Heston et al. Prostrate (US), 1982, 3 (4), p383-9

Stefanelli et al, biochemical journal (England), Apr. 1, 2001. 355 (pt. 1), p199-206.

Lopez et al., biocell: official journal of the sociedades latinoamericanas de microscopia electronica... et. al. 9Argentina), Dec. 1999, 23 (3), p223-8.

Schipper et al. seminars in cancer biology (US), feb. 2000, 10 (1), p55-68.

Nilsson et al., biochemical journal (England) Mar. 15, 2000, 346 Pt. 3, p699-704.

giuseppina monti m. et al., biochemical and biophysical research commun. (US), Apr. 13, 1999, 257 (2), p460-5.

ratasirayakorn et al, j. of periodontology feb. 1999, 70 (2), p179-84

stabellini et al., Experimental and molecular parhology (US), 1997, 64 (3), p147-55.

Sparapani et al, experimental neurology (US), nov. 1997, 148 (1), p157-66..

Dhalluin et al., carcinogenesis (Eng.), Nov. 1997, 18 (11), p2217-23.

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Inhibition of ornithine decarboxylase by α-difluoromethylornithine induces apoptosis of HC11 mouse mammary epithelial cells*

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Summary. The effect of α-difluoromethylornithine (DFMO) on the apoptosis of HC11 mouse mammary epithelial cells was investigated. The involvement of reactive oxygen species (ROS) and Bcl-2 protein in the mechanism of apoptosis induced by ornithine decarboxylase (ODC) inhibition was also assessed. DFMO (0.1, 1 and 5 mM) induced apoptosis of HC11 cells in dose- and time-dependent manner. Apoptosis manifests itself with morphological features like: cell shrinkage, condensation of chromatin, pyknosis and fragmentation of nucleus, followed by secondary necrosis (putrosis). The decrease in the nuclear DNA contents appearing as the hypodiploidal peak sub-G_1 in the DNA histogram was not dependent on the presence of prolactin (5 μg/ml) in DFMO-treated cultures. Apoptosis induced by ODC inhibition was associated with a rapid increase in ROS concentration in HC11 cells observed within 1 h after DFMO treatment. The down-regulation of Bcl-2 as a decrease in cell number expressing *bcl-2* and a lowered Bcl-2 protein content in cells expressing this protooncogene was also noted. The administration of putrescine (50 μM) lowered the number of early-apoptotic, late-apoptotic and necrotic cells. Moreover, it increased the number of cells expressing *bcl-2*. In conclusion, the disturbance of cellular polyamine homeostasis by inhibition of their synthesis enhances mammary epithelial cell susceptibility to apoptosis. It may occur in the mammary gland at the end of lactation, when the depletion of circulating lactogenic hormones and activation of intra-mammary apoptogenic factors expression take place.

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Keywords: Amino acids – Polyamines – Ornithine decarboxylase – α-Difluoromethylornithine – Apoptosis – Bcl-2 – Mammary epithelial cells

Introduction

Apoposis – programmed cell death is responsible for the loss of mammary epithelial cells in the course of morphogenesis (Humphreys et al., 1996) and involution of mammary gland after weaning in rodents (Quarrie et al., 1995, 1996) and during drying off in ruminants (Wilde et al., 1997). The depletion of circulating lactogenic hormones (prolactin, GH), milk stasis and intramammary apoptogenic agents (Feedback Inhibitor of Lactation, TGF-β_1) are involved in the mechanism of mammary epithelial cells apoptosis. It is generally accepted that polyamines are implicated in signal transduction pathways of hormones (prolactin, insulin, glucocorticoids) and growth factors (EGF, IGF-I, IGF-II, PDGF, TGF-α), which are the regulators of mammary epithelial cells proliferation, differentiation and milk protein synthesis. The one set of lactation is associated with the highest activity of ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) and the highest concentration of putrescine, spermidine and spermine in colostrum and milk of ruminants (Motyl et al., 1995, Motyl et al., 1996a, Płoszaj et al., 1997). The earlier studies proved that the induction of ODC and an increase in polyamine synthesis are the important early events in the lactogenic effect of prolactin (Rillema, 1976, 1980; Rillema et al., 1977). Our recent study indicates that prolactin is not only lactogenic, but also an antiapoptotic hormone in HC11 mouse mammary epithelial cells (Płoszaj et al., 1998). In contrast to prolactin, TGF-β_1 is an antiproliferative and apoptogenic factor for mammary epithelial cells (Motyl et al., 1998). The action of this cytokine is associated with the inhibition of expression and activity of ODC and SAMDC in leukaemic cells (Motyl et al., 1993, 1996b). Interestingly, phorbol ester (TPA) and orotic acid being potent ODC inducers prevent TGF-β_1-evoked apoptosis (Grzelkowska et al., 1995, Motyl et al., 1996b). It has been also shown that inhibition of ODC by α-difluoromethylornithine (DFMO) induces apoptosis of L1210 leukaemic cells, which manifests itself in internucleosomal fragmentation of DNA (Motyl et al., 1996b). All these data indicate that polyamines are the mediators of survival signals brought by hormones and growth factors. Conversely, the depletion of intracellular polyamines is associated with growth inhibition and apoptosis. We assume that changes in homeostasis of polyamines in mammary epithelial cells physiologically occuring in lactation cycle may affect their susceptibility to apoptosis. Using a model of HC11 mouse mammary epithelial cells culture we have confirmed this assumption, showing that inhibition of ODC by DFMO induces apoptosis regardless of the presence or absence of prolactin in extracellular environment. This effect was associated with an increase in reactive oxygen species (ROS) concentration and down-regulation of Bcl-2, being the physiological inhibitor of apoptosis. Exogenous putrescine increased *bcl-2* expression, and lowered the extent of DFMO-induced apoptosis.

Material and methods

Media and reagents

DMEM powdered medium lacking L-glutamine, L-glutamine, phosphate buffered saline (PBS), fetal calf serum (FCS), fungizone, gentamycin sulphate were obtained from Gibco BRL (Paisley, Scotland). FITC-conjugated monoclonal anti-Bcl-2 antibodies and FITC-conjugated monoclonal anti-G_1 antibodies were supplied by DAKO (Glostrup, Denmark). 6-Carboxy-2',7'-dichloro-dihydrofluorescein diacetate, di(acetoxymethyl ester) (C-DCDHF-DA), Hoechst 33342, 4',6-diamidino-2-phenylindole (DAPI), were from Molecular Probes Europe BV (Leiden, The Netherlands). Putrescine (PTR), ovine prolactin (PRL), bovine insulin, murine epidermal growth factor (EGF), propidium iodide, sulforhodamine, and other chemicals were provided by Sigma Chemical Corp. (St. Louis, MO, USA). α-Difluoromethylornithine (DFMO) was kindly supplied by the Merrel Down Research Institute (Cincinati, OH, USA). Sterile conical flasks and sterile disposable pipettes were purchased from Nunc Inc., (Naperville, IL, USA).

Cell culture

The mouse mammary epithelial HC11 cell line was supplied by Dr. Bernd Groner (Institute for Experimental Cancer Research, Tumor Biology Centre, Frieburg, Germany). Cell cultures were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 0.2% (w/v) L-glutamine, gentamicin (50μg/ml), fungizone (2.5μg/ml), bovine insulin (5μg/ml), EGF (10 ng/ml) in an atmosphere of 5% CO_2/95% humidified air at 37°C, and routinely subcultured every 2 or 3 days. For the measurement of reactive oxygen species (ROS) generation, *bcl-2* expression and percentage of apoptotic cells, cultures were preincubated for 24 h in the absence of EGF. Then the cells were exposed to EGF-deficient medium containing DFMO (0.1, 1 and 5 mM), DFMO with PTR (50μM) in combination with or without PRL (5μg/ml) as indicated for particular experiments.

Cell labelling for determination of apoptosis

In this method, the cells (stored in 70% ethanol) were stained with 1μg/ml DAPI (DNA labelling) and 10μg/ml sulforhodamine 101 (protein labelling), dissolved in 10 mM piperazine-N,N-bis-2-ethanesulfonic acid buffer, containing 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% Triton X-100 (pH 6.8). The fluorescence of individual cells was measured with Beckton Dickinson (San Jose, CA, USA) FACS VANTAGE flow cytometer.

Microscopy

Morphological evaluation of apoptosis was performed using: 1) the combination of propidium iodide followed by Hoechst 33342 staining to distinguish live, necrotic, early- and late-apoptotic cells, 2) labelling of DNA and protein with DAPI and sulforhodamine, respectively. Microscopy and microphotography was performed with a BX-60 Olympus fluorescence microscope equipped with PM20 automatic photomicrographic system (Olympus Optical Corp. Ltd., Tokyo, Japan).

Fluorescent measurement of intracellular peroxides

To assess the intracellular concentration of peroxides generated in DFMO-treated cultures, the oxidation-sensitive fluorescent marker C-DCDHF-DA was used. This method was applied to various models of ROS – mediated apoptosis, and as a marker of antioxidative action of Bcl-2 protein (Hockenbary et al., 1993; Satoh et al., 1996). In the present study, control (10% FCS/DMEM) and DFMO-treated (5 mM) cells (5×10^5/ml) were loaded with 5μM C-DCDHF-DA (dissolved in dimethylsulfoxide at 2000×) for

30 min. at 37°C prior to the measurement. The cells (1×10^4) were analysed with a Becton Dickinson FACS VANTAGE flow cytometer with excitation and emission settings of 488 nm and 530 nm, respectively.

Bcl-2 labelling

After centrifugation at 200 g for 10 min. the cells (1.5×10^6) were resuspended in 100 μl of Hank's balanced salt solution and transferred to Eppendorf centrifuge tubes. Next, 1 ml of parafomaldehyde 0.25% (v/v) was slowly added with simultaneous mixing on vortex. Afterwards, the cells were incubated in the dark for 15 min. at room temperature (20°C). The cells were centrifuged at 300 g for 5 min. (20°C), then the supernatant was discarded and the cell pellet washed twice with PBS. After subsequent centrifugation (300 g for 5 min., 20°C) 1 ml of ice cold methanol 70% (v/v) was added, and the cells were vortexed. The suspension of cells was incubated in the refrigerator for 60 min. (4°C). After centrifugation (300 g for 5 min., 4°C) the supernatant was aspired and discarded, the cells were washed in PBS and centrifuged as described above. An aliquot of 10 μl of FITC-conjugated monoclonal anti-Bcl-2 antibody or FITC-conjugated mouse anti-IgG1 antibody solution (negative control) was added to the experimental sample. The cell suspension was gently vortexed. Then, the cells were incubated for 45 min. at 4°C. 1 ml of aliquot of 2% FCS/PBS was added and after mixing the cells were centrifuged (300 g for 5 min., 4°C) and the supernatant discarded. The cells were suspended in 300 μl of PBS, stained with 1.5 ml of DAPI (1 μg/ml the solution consisting of 10 mM PIPES, 2 mM $MgCl_2$ and 1% Triton X-100) and analysed by flow cytometry.

Flow cytometry

We used a Becton-Dickinson FACS VANTAGE flow cytometer equipped with two-wavelength laser system Innova-Enterprise: blue (488 nm) and UV (351 nm). DAPI was excited in UV range, and emitted blue (424 nm) fluorescence light, whereas FITC was excited in blue (488 nm) and emitted green light (530 nm). Both exciting beams are spatially separated (distance = 150 μm) so fluorescence spectra can be easily separated using half-height mirrors and appropriate dichroic mirrors. Data acquisition and analysis were done with B-D software (Cell-Quest). The cell cycle analysis was done using Mac-Cycle software (Phoenix Flow Systemy, San Diego, CA, USA). 1×10^4 cells were, as a rule, collected and analysed in each sample.

Statistical evaluation

The results were statistically evaluated with ANOVA and Tukey's multiple range test. The probability of differences at the level $P \leq 0.05$ was considered as significant, and at the level $P \leq 0.01$ as highly significant. The correlations were calculated with Prism™ version 2.00 software (GraphPad Software Inc., San Diego, CA, USA).

Results

The qualitative and quantitative analysis of apoptosis in HC11 mouse mammary epithelial cells was performed by fluorescence microscopy and flow cytometry, respectively. Morphological evaluation of DFMO-treated cultures showed a high number of cells with characteristic features of apoptosis like: cell shrinkage, chromatin condensation, pyknosis and fragmentation of nuclei followed by secondary necrosis – putrosis. The greek term *putrosis* (corresponding to the rotting of leaves after they have fallen) was proposed by Vermes et al. (1997) to indicate the postapoptotic status

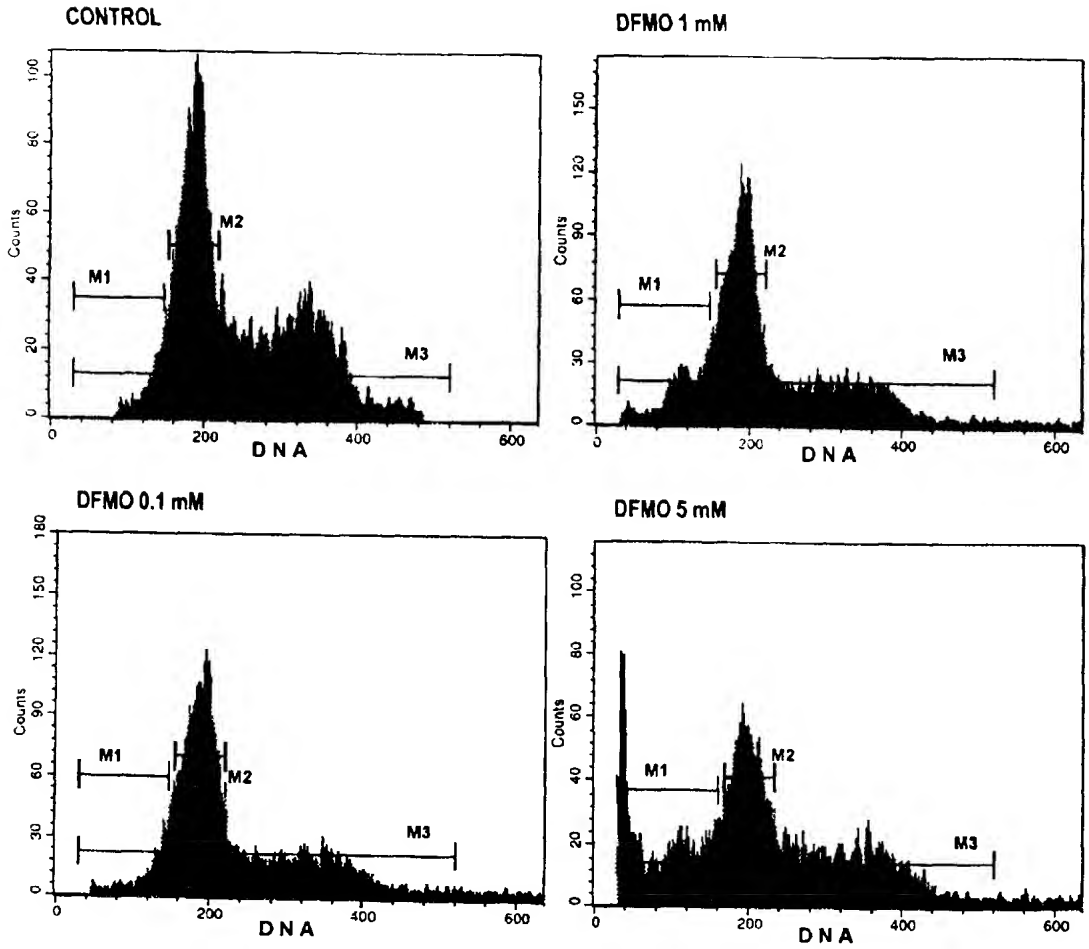

Fig. 1. DNA content frequency distribution histogram of control (10% FCS/DMEM) and DFMO-treated (0.1, 1 and 5 mM) HC11 mouse mammary epithelial cells for 24 h. DNA was stained with DAPI. Region M1 represents apoptotic cells (cells with lowered DNA content). Presented histograms are representative of three separate experiments

primarily found under *in vitro* culture conditions where no phagocytes are present.

For the quantification of apoptotic cell number, we stained DNA with DAPI, and measured the hypodiploid peak in a DNA content frequency histogram (Fig. 1). The increasing concentrations (0.1, 1 and 5 mM) of DFMO in the incubation medium evidently enlarged the sub-G_1 region, corresponding to apoptotic (and secondary necrotic) cells with lowered DNA content, after 24 h of incubation. The average number of apoptotic cells in cultures treated with the highest concentration of DFMO (5 mM) exceeded 30% (Fig. 2). The administration of PTR (50 μM) diminished the apoptotic effect of DFMO, however statistically significant only at 5 mM concentration of ODC inhibitor (Fig. 2).

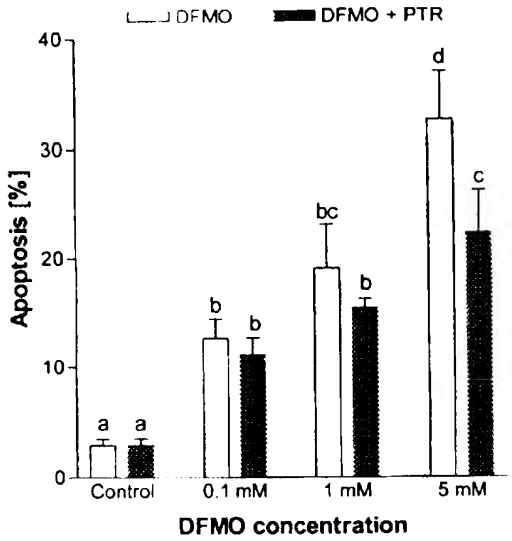

Fig. 2. Effect of increased concentration of DFMO (0.1, 1 and 5 mM) in culture medium (10% FCS/DMEM) and administration of putrescine (50μM) on apoptotic cell number after 24 h. Each value represents the mean (±SEM) of three separate experiments. Means marked with different superscript letters differ significantly ($P < 0.05$)

The discrimination between live, early apoptotic, late apoptotic and necrotic cells was possible with the use of combined cell staining with propidium iodide and Hoechst 33342. The accessibility of these fluorochromes to nuclear DNA depends on cell membrane integrity. Nuclei of live and early apoptotic cells were labelled with different intensity by HO 33342 (blue fluorescence), whereas nuclei of late apoptotic and necrotic cells were labelled mainly with propidium iodide (red fluorescence). DFMO (5 mM) increased the number of early and late apoptotic as well as necrotic cells during 48 h of culturing (Fig. 3). The most dynamic was the gain of late apoptotic cells reaching 50% of total cell population after 48 h. Exogenous PTR (50μM) reduced the intensity of all forms of cell deaths.

Prolactin decreased the extent of spontaneous apoptosis in control culture (10% FCS/DMEM), however it did not prevent DFMO-induced apoptosis of HC11 cells (Fig. 4). Similarly to the preceding experiments (Figs. 2 and 3), PTR significantly reduced the number of apoptotic cells in DFMO-treated cultures both in the presence or absence of prolactin.

The administration of DFMO (5 mM) to HC11 cell culture increased ROS generation within the cells reaching the peak after 60 min. (Fig. 5c). Formation of ROS manifested itself as yellow-green fluorescence of the oxidation-sensitive derivative of fluoresceine (Fig. 5, compare a and b).

The inhibition of ODC by DFMO significantly decreased the number of HC11 cells expressing bcl-2 protooncogene. It was shown on fluorescence histogram where decrease in the peak of cells with specific anti-Bcl-2

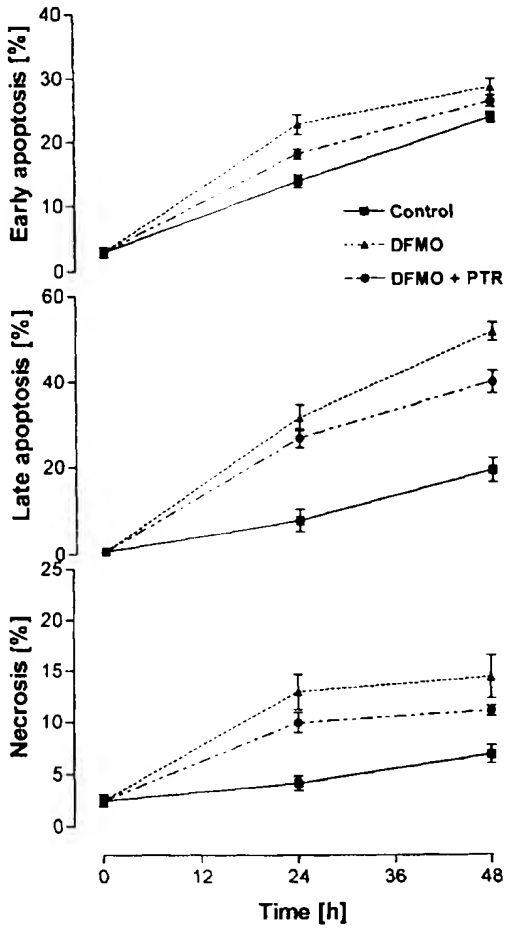

Fig. 3. Influence of DFMO (5mM) and DFMO with putrescine (50μM) on number of early apoptotic, late apoptotic and necrotic cells during 48h (means ± SEM). Control cells were cultured in 10% FCS/DMEM. Cells were stained with propidium iodide and HO33342 (see Material and methods)

fluorescence (region M2) was accompanied by an increase in the cell number with non-specific anti-Bcl-2 fluorescence (regions M1 and M3) (Fig. 6a). The increase in DFMO concentration in the medium decreased the number of cells expressing *bcl-2* on average from 83% in the control culture to 52% at 5mM of DFMO after 24h (Fig. 6b).

The relative Bcl-2 protein level in the cell, calculated by the ratio of the median of specific to non-specific fluorescence peak, decreased in DFMO-treated cultures. The negative and nonlinear correlation ($r = 0.41$, $P \leq 0.05$) between the concentration of DFMO in the medium and relative Bcl-2 level in HC11 cells was calculated (Fig. 7).

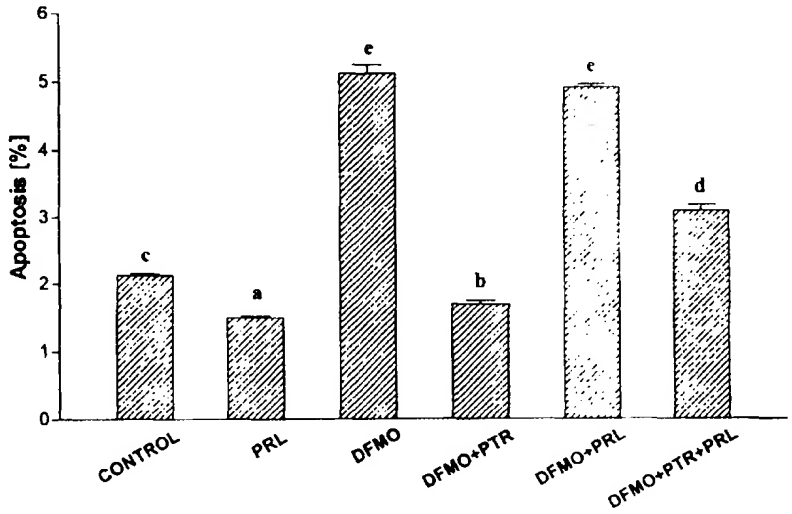

Fig. 4. Effect of DFMO (5mM) and DFMO with putrescine (50μM) on apoptotic cell number in prolactin-treated (5μg/ml) and non-treated cells for 24h. Each bar represents the mean (±SEM) of three cultures. Means described with different superscript letters differ significantly ($P < 0.05$)

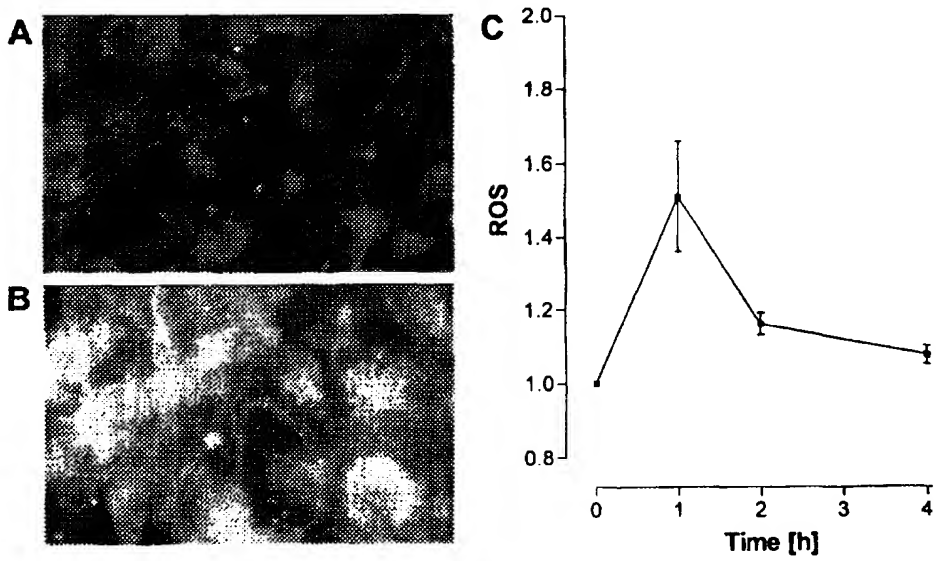

Fig. 5. Effect of DFMO (5mM) on ROS generation in HC11 cells. C-DCDHF-DA-derived fluorescence in control (**A**) (200×) and DFMO-treated (**B**) (200×) cells after 60min. The ratio of experimental (DFMO) to control (10% FCS/DMEM) values of medians of C-DCDHF-DA-derived fluorescence peaks (**C**)

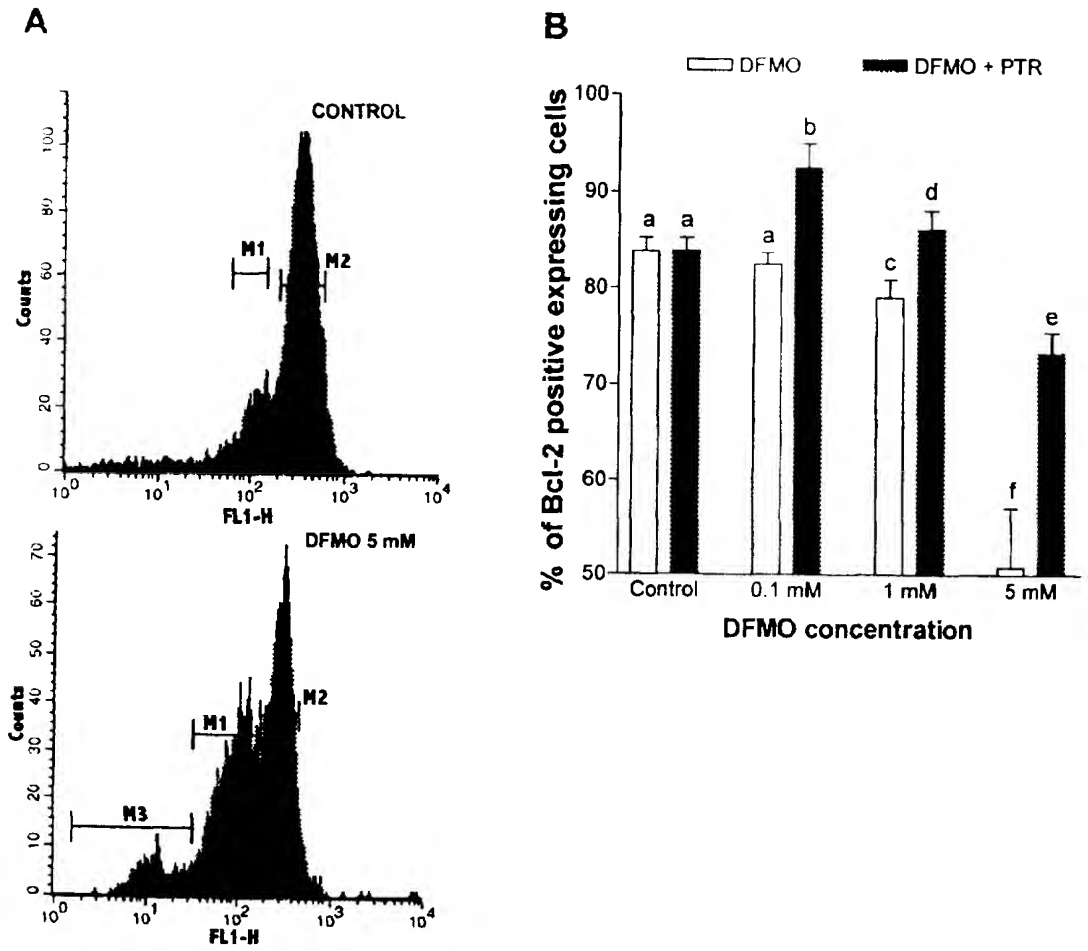

Fig. 6. **A** Histograms of specific (region M2) and nonspecific (regions M1 and M3) anti-Bcl-2 fluorescence of HC11 cells cultured in control (10% FCS/DMEM) and DFMO-supplemented (5 mM) medium for 24 h. **B** Effect of increasing concentration of DFMO (0.1, 1 and 5 mM) with or without putrescine (50 μM) on number of cells expressing *bcl-2* protooncogene (cells with specific anti-Bcl-2 fluorescence – see Fig. 6A). Each bar represents mean (±SEM) of three different experiments. Means bearing different superscript letter differ significantly ($P < 0.05$)

Discussion

The present study has proved that polyamines, apart from their role in proliferation, differentiation, maturation, transformation and overall cellular metabolism, protect the cell against apoptogenic stimuli. The inhibition of ODC activity – a key enzyme in polyamine pathway by DFMO induced apoptosis of HC11 mouse mammary epithelial cells. Characteristic morphological features of apoptosis like: cell shrinkage, condensation of chromatin, fragmentation of nucleus, and formation of apoptotic bodies were

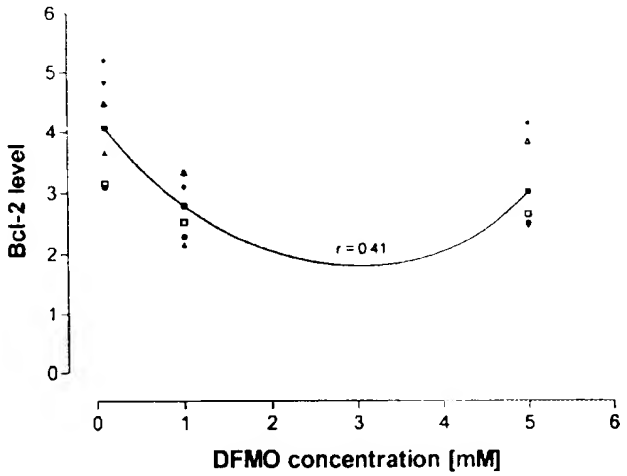

Fig. 7. Relationship between the concentration of DFMO and relative Bcl-2 level in cells expressing this protooncogene (region M2 – Fig. 6A). Bcl-2 level was calculated as a median of specific fluorescence region: median of nonspecific fluorescence region (negative control) ratio. The correlation was calculated from three separate experiments using GraphPad Prism™ version 2.0 software. Regression equation was: Y = Span1*exp(−K1*X) + Span2*exp(−K2*X) + Plateau. Correlation coefficient was statistically significant ($P < 0.05$)

observed. A lowered DNA content in apoptotic cells manifested itself in formation of sub-G1 peak in DNA frequency distribution histograms (Fig. 1). The apoptogenic effect of DFMO was both dose- (Fig. 2) and time- (Fig. 3) dependent. Since the cell culture is a closed system, apoptotic cells do not undergo phagocytosis but secondary necrosis. These cells can be distinguished by simultaneous staining with propidium iodide and Hoechst 33342 (Fig. 3). DFMO-induced apoptosis was observed also in L1210 leukaemic cells as a characteristic "DNA ladder", which was a result of internucleosomal DNA cleavage for fragments that are multiples of 180-200 bp (Motyl et al., 1996b). Noteworthy is the fact that the antiapoptotic effect of prolactin in HC11 mouse mammary epithelial cells (Płoszaj et al., 1998) dissapeared in the presence of DFMO (Fig. 4). It confirms the earlier reports indicating that ODC/polyamines system is a part of prolactin signal transduction pathway. DFMO blocks not only prolactin effects, but also survival signals induced by other hormones, cytokines and growth factors (Blachowski et al., 1994, Kasterka et al., 1994).

The mechanism of apoptosis evoked by inhibition of ODC is probably associated with deficiency in intracellular polyamines. It could be confirmed by the protective effect of exogenous PTR, which diminished the intensity of apoptosis induced by DFMO (Figs 2, 3 and 4). It has been shown that the uptake and utilization of extracellular PTR increase when ODC is inhibited (Redgate et al., 1993). There are several recent reports indicating that depletion of intracellular polyamines induces apoptosis in many types of cells.

The antitumour effect of a polyamine biosynthetic pathway inhibitor MGBCP is associated with induction of apoptosis in human hepatocellular carcinoma HEP-1 and human breast cancer MRK-nu-1 cell lines and this effect is partially prevented by exogenous spermine (Hashimoto et al., 1999; Kaneko et al., 1998). The superinduction of polyamine catabolic enzyme SSAT or inhibition of PAO triggers or prevents apoptosis, respectively (Ha et al., 1997). It was also demonstrated that the inhibition of the cytosolic 26S proteasome function preserves ODC activity and prevents thymocytes from undergoing apoptosis after dexamethasone treatment (Grassili et al., 1998). However, it should be mentioned that polyamines are not only survival but also apoptogenic agents in abnormally high concentrations. Over-accumulation of natural and synthetic polyamines, but not putrescine, rapidly induces apoptosis of L1210 leukaemic cells (Poulin et al., 1995). It has been shown that the anti-neoplastic effects of bis(ethyl) polyamine analogues may be due to the induction of apoptosis in sensitive tumour cells (Hu and Pegg, 1997). The consequences of intracellular polyamine deficiency may be as many-sided as their functions in the cell. They can be rapid and short-lasting as well as slow and long-lasting. The first category is represented by the increase in intracellular ROS concentration observed within 1 h after DFMO administration (Fig. 5). ROS are important candidates for inducers of apoptosis in DFMO-treated HC11 cells. Our earlier study showed that the influence of peroxyl radical initiator – AAPH on L1210 leukaemic cells is associated with intracellular ROS generation, lipid peroxidation and induction of apoptosis (Zimowska et al., 1997). Since polyamines stabilize basement and organellar membranes (Pavlovic et al., 1992), their deficiency may lead to the disruption of transmembrane potential, increased permeability transition and escape of ROS from cellular sources of their generation (eg. mitochondria, endoplasmic reticulum). Oxidative stress damages nuclear and mitochondrial DNA by oxidation of sugar and base residues, which in turn, triggers a cascade of events leading to the cell death e.g. activation of *p53* gene (guardian of genome), up-regulation of Bax subfamily proteins (promoters of apoptosis) and down-regulation of Bcl-2 subfamily proteins (inhibitors of apoptosis), activation of caspases and DNases (executors of apoptosis) (Kroemer, 1997; Green and Reed, 1998). Polyamine depletion by DFMO was accompanied by a significant increase in *p53* expression, and polyamines given together with DFMO completely prevented this effect (Li et al., 1999). Polyamines are known as biologically important antioxidants (Ha et al., 1998; Løvaas 1995, Pavlovic et al., 1992) and may protect cells from apoptosis in these cases, where ROS are mediators in apoptotic cell death (Sarafian and Bredesen, 1994). The mechanism of this protection can be due to prevention of ROS generation and release (Løvaas 1995), ability to ROS scavenge (Farbiszewski et al., 1996; Ha et al., 1998) and sterical interactions of polyamines with DNA (Muscari et al., 1995). The depletion of intracellular polyamines can cause the alteration of DNA supercoiling and increase sensitivity of molecular DNA to digestion by endonucleases activated in the course of the apoptotic process (Orrenius, 1995). It has been shown that spermine can prevent DNA fragmentation and

apoptosis in thymocytes exposed to glucocorticoids and Ca^{2+} ionophores (Brüne et al., 1991) as well as in irradiated LY-TH lymphoma cells (Meyn et al., 1993).

Inhibition of ODC by DFMO decreased the number of HC11 cells expressing *bcl-2* (Fig. 6) and Bcl-2 protein level in cells expressing this protooncogene (Fig. 7) indicating an enhanced cell susceptibility to apoptosis. Exogenous PTR increased the extent of *bcl-2* expression in DFMO treated cells, which could be either a direct effect due to involvement of polyamines in transcription and translation of *bcl-2* protooncogene and/or indirect due to the antioxidative properties of polyamines and blocking a chain of reactions leading to down-regulation of Bcl-2. In spite of intensive studies the molecular mechanism of antiapoptotic action of Bcl-2 protein is still unclear. The places of its action are membranes of mitochondria, nucleus and endoplasmic reticulum – organelles, being a source of ROS in the cell. It is suggested that Bcl-2 may function as a scavenger of free radicals, acting in the sphere between generation of peroxides and peroxidation of cellular lipids (Hockenbery et al., 1993), and as a regulatory protein, protecting against the decrease in antioxidant enzymes activity (Baker et al., 1996). Overexpression of *bcl-2* protects against the release of Apoptosis Inducing Factor (Kroemer, 1997) and cytochrome c from intermembrane space of mitochondria (Liu et al., 1996) which are responsible for the activation of caspases cascade and the triggering of the destructive phase of apoptosis.

In conclusion, the maintenance of polyamines homeostasis is indispensable for cell survival. The disturbance of this homeostasis by inhibition of their synthesis enhances mammary epithelial cell susceptibility to apoptosis. From a physiological and practical point of view it may occur in the mammary gland at the end of lactation when depletion of lactogenic hormones secretion with simultaneous activation of intramammary apoptogenic factors expression occur.

References

Baker AM, Briehl MM, Dorr R, Powis G (1996) Decreased antioxidant defence and increased oxidant stress during dexamethasone-induced apoptosis: bcl-2 prevents the loss of antioxidant enzyme activity. Cell Death Differ 3: 207–213

Blachowski S, Motyl T, Grzelkowska K, Kasterka M, Orzechowski A, Interewicz B (1994) Involvement of polyamines in epidermal growth factor (EGF), transforming growth factor (TGF)-α and -β_1 action on culture of L6 and fetal bovine myoblasts. Int J Biochem 26: 891–897

Brüne B, Hartzell P, Nicotera P, Orrenius S (1991) Spermine prevents endonuclease activation and apoptosis in thymocytes. Exp Cell Res 195: 323–329

Farbiszewski R, Bielawska A, Szymanska M, Skrzydlewska E (1996) Spermine partially normalizes in vitro antioxidant defense potential in certain brain regions in transiently hypoperfused rat brain. Neurochem Res 21/12: 1497–1503

Grassilli E, Benatti F, Dansi P, Giammarioli AM, Malorni W, Franceschi C, Desiderio MA (1998) Inhibition of proteasome function prevents thymocyte apoptosis: involvement of ornithine decarboxylase. Biochem Biophys Res Commun 250/2: 293–297

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309–1310

Grzelkowska K, Motyl T, Malicka E, Ostrowski J, Trzeciak L, Filipecki M (1995) Effect of orotic acid on TGF-β_1 induced growth inhibition of L1210 leukemic cells. Int J Hematol 61: 23–33

Ha HC, Woster PM, Yager JD, Casero RA Jr (1997) The role of polyamine catabolism in polyamine analogue-induced programmed cell death. Proc Natl Acad Sci 94/21: 11557–11562

Ha HC, Sirisoma NS, Kuppusamy P, Zweier JL, Woster PM, Casero RA Jr (1998) The natural polyamine spermine functions directly as a free radical scavenger. Proc Natl Acad Sci 95/19: 11140–11145

Hashimoto Y, Hibasami H, Tamaki S, Kamei A, Ikoma J, Kaito M, Imoto I, Watanabe S, Nakashima K, Adachi Y (1999) Induction of apoptotic cell death in human hepatocellular carcinoma SK-HEP-1 cells by a polyamine synthesis inhibitor, methylglyoxal bis(cyclopentylamidinohydrazone). Anticancer Drugs 10/3: 323–327

Hockenbery DM, Oltvai ZN, Yin X-M, Milliman CL, Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75: 241–251

Hu RH, Pegg AE (1997) Rapid induction of apoptosis by deregulated uptake of polyamine analogues. Biochemical J 328: 307–316

Humphreys RC, Krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S, Reed JC, Rosen JM (1996) Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development 122: 4013–4022

Kaneko H, Hibasami H, Mori K, Kawarada Y, Nakashima K (1998) Apoptosis induction in human breast cancer MRK-nu-1 cells by a polyamine synthesis inhibitor, methylglyoxal bis(cyclopentylamidinohydrazone) (MGBCP). Anticancer Res 18/2A: 891–896

Kasterka M, Motyl T (1994) The role of polyamines on cytokines and growth factors action in K562 leukemic cells. In: Proceedings of 3rd Symposium: Molecular and physiological aspects of regulatory processes (polish edition). pp 121–123

Kroemer G (1997) The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nature Med 3: 614–620

Li L, Li J, Rao JN, Li M, Bass BL, Wang JY (1999) Inhibition of polyamine synthesis induces p53 gene expression but not apoptosis. Am J Physiol 276(4 Pt 1): C946–C954

Liu X, Kim CM, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 12: 147–157

Løvaas E (1995) Hypothesis: spermine may be an important epidermal antioxidant. Med Hypoth 45: 59–67

Meyn RE, Stephens LC, Voehringer DW, Story MD, Mirkovic N, Milas L (1993) Biochemical modulation of radiation-induced apoptosis in murine lymphoma cells. Radiat Res 136: 327–334

Motyl T, Kasterka M, Grzelkowska K, Blachowski S, Sysa P (1993) TGF-β_1 inhibits polyamine biosynthesis in K 562 leukemic cells. Ann Hematol 67: 285–288

Motyl T, Płoszaj T, Wojtasik A, Kukulska W, Podgurniak M (1995) Polyamines in cows and cow's milk. Comp Biochem Physiol 111B/3: 427–433

Motyl T, Płoszaj T, Ryniewicz Z (1996a) Polyamines in colostrum and milk of farm animals. In: Proceedings of 5th International symposium: Hormones and bioactives substances in milk. Smolenice, The Slovak Republic

Motyl T, Kasterka M, Grzelkowska K, Ostrowski J, Filipecki M, Malicka E, Płoszaj T (1996b) Phorbol ester (12-0-tetradecanoyl-phorbol 13-acetate) prevents ornithine decarboxylase inhibition and apoptosis in L1210 leukemic cells exposed to TGF-β_1. Int J Biochem Cell Biol 28/12: 1327–1335

Motyl T, Waręski P, Płoszaj T, Janczewska S, Orzechowski A, Ryniewicz Z (1998) Involvement of Bax and Bcl-2 protein in regulation of mammary epithelial cells apoptosis. BASE 2: L/M2

Muscari C, Guarnieri C, Stefanelli C, Giaccari A, Caldarera CM (1995) Protective effect of spermine on DNA exposed to oxidative stress. Mol Cell Biochem 144: 125–129

Orrenius S (1995) Apoptosis: molecular mechanisms and implications for human disease. J Int Med 237: 529–536

Poulin R, Pelletier G, Pegg AE (1995) Induction of apoptosis by excessive polyamine accumulation in ornithine decarboxylase-overproducing L1210 cells. Biochemical J 311: 723–727

Pavlovic DD, Uzunova P, Galabova T, Peneva V, Sokolova Z, Bjelakovic G, Ribarov S (1992) Polyamines as modulators of lipoperoxidation. Gen Physiol Biophys 11: 203–211

Płoszaj T, Motyl T, Orzechowski A, Zimowska W, Waręski P, Skierski J, Zwierzchowski L (1998) Antiapoptotic action of prolactin is associated with up-regulation of Bcl-2 protein in HC11 mouse mammary epithelial cells. Apoptosis 3: 295–304

Płoszaj T, Ryniewicz Z, Motyl T (1997) Polyamines in goat's colostrum and milk. Comp Biochem Physiol 118B/1: 45–52

Quarrie LH, Addey CVP, Wilde CJ (1995) Apoptosis in lactating and involuting mouse mammary tissue. Cell Tissue Res 281: 413–419

Quarrie LH, Addey CVP, Wilde CJ (1996) Programmed cell death during mammary tissue involution induced by weaning, litter removal, and milk stasis. J Cellular Physiol 168: 559–569

Redgate ES, Grudziak A, Floyd KL, Deutsch M, Boggs SS (1993) Effect of D,L-α-difluoromethylornithine (DFMO) enhanced [^{3}H] putrescine uptake on 9L tumor cell growth and colony forming efficiency. Int J Radiat Oncol Biol Phys 25: 639–646

Rillema JA (1976) Action of prolactin on ornithine decarboxylase activity in mammary gland explants of mice. Endocrine Res Commum 3/5: 297–305

Rillema JA, Linebaugh BE, Mulder JA (1977) Regulation of casein synthesis by polyamines in mammary gland explants of mice. Endocrinology 100: 529

Rillema JA, Schneider-Kuznia M (1980) Observations on the early actions of prolactin on RNA and casein synthesis in mouse mammary gland explants. Hormone Res 12: 218–223

Sarafian TA, Bredesen DE (1994) Invited commentary: is apoptosis mediated by reactive oxygen species? Free Radic Res 21/1: 1–8

Satoh T, Sakai N, Enokido Y, Uchiyama Y, Hatanaka H (1996) Survival factor-insensitive generation of reactive oxygen species induced by serum deprivation in neuronal cells. Brain Res 733: 9–14

Vermes I, Haanen C, Richel DJ, Schaafsma MR, Kalsbeek-Batenburg E, Reutelingsperger CP (1997) Apoptosis and secondary necrosis of lymphocytes in culture. Acta Haematol 98: 8–13

Wilde CJ, Quarrie LH, Tonner E, Flint DJ, Peaker M (1997) Mammary apoptosis: physiological regulation and molecular determinants. Livest Prod Sci 50/1–2: 29–37

Zimowska W, Motyl T, Skierski J, Bałasińska B, Płoszaj T, Orzechowski A, Filipecki M (1997) Apoptosis and Bcl-2 protein changes in L1210 leukaemic cells exposed to oxidative stress. Apoptosis 2: 529–539

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